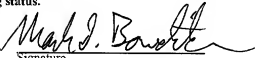


FORM PTO-1590		U.S. Department of Commerce Patent and Trademark Office	Attorney's Docket No. 2577-127
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>			U.S. Application No. (if known, see 37 CFR 1.5) <b>10/019661</b>
INTERNATIONAL APPLICATION NO. PCT/SG/99/00128	INTERNATIONAL FILING DATE November 17, 1999	PRIORITY DATE CLAIMED July 2, 1999	
<b>TITLE OF INVENTION</b> GLOBAL REGULATORS OF BACTERIAL PATHOGENIC GENES AS TARGETS FOR ENGINEERING DISEASE RESISTANCE			
<b>APPLICANT(S) FOR DO/EO/US</b> Lian-Hui, Zhang; Yihu, Dong; JinLing, Xu			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US) 6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input checked="" type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
<b>ITEMS 11. TO 20. below concern other document(s) or information included:</b>			
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input type="checkbox"/> A <b>FIRST</b> preliminary amendment. 14. <input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input type="checkbox"/> Other items or information:			

U.S. APPLICATION NO. <b>10/019661</b>		INTERNATIONAL APPLICATION NO. PCT/SG99/00128		ATTORNEY DOCKET NO. 2577-127	
17. [X] The following fees are submitted: <b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report Not Prepared by EPO or JPO ..... \$ 1,040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report has been prepared by the EPO or JPO ..... \$ 890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$ 740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$ 710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$ 100.00  <div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>				<b>CALCULATIONS</b>	<b>FTO USE ONLY</b>
				<div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>	
Surcharge of \$130.00 for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
<b>Claims</b>	<b>Number Filed</b>	<b>Number Extra</b>	<b>Rate</b>		
Total Claims	34 -20 =	14	X \$18.00	\$ 252	
Independent Claims	6 -3 =	3	X \$84.00	\$ 252	
Multiple dependent claim(s) (if applicable)			+ \$280.00	\$ 280	
<b>TOTAL OF ABOVE CALCULATIONS =</b>				\$ 1824	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	
<b>SUBTOTAL =</b>				\$	
Processing fee of \$130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
<b>TOTAL NATIONAL FEE =</b>				\$ 1824	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				+	\$
<b>TOTAL FEES ENCLOSED =</b>				\$ 1824	
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a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. 02-2135 in the amount of \$ 1824 to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-2135. A duplicate copy of this sheet is enclosed. <b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a)          or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO:  <div style="display: flex; justify-content: space-between;"> <div> <b>Customer No. 6449</b>             Mark I. Bowditch            Rothwell, Figg, Ernst &amp; Manbeck            555 13th St., N.W.            Washington, D.C. 20004            Phone: 202/783-6040         </div> <div style="text-align: right;">             Signature             Mark I. Bowditch            Name             40,315            Registration Number         </div> </div>					

## TITLE OF THE INVENTION

GLOBAL REGULATORS OF BACTERIAL PATHOGENIC GENES; BACTERIAL AUTOINDUCER  
INACTIVATION PROTEIN, AS TARGETS FOR ENGINEERING DISEASE RESISTANCE

## CROSS-REFERENCES TO RELATED APPLICATIONS

Not applicable.

STATEMENT REGARDING FEDERALLY SPONSORED  
RESEARCH OR DEVELOPMENT

Not applicable.

## BACKGROUND OF THE INVENTION

## 1. Field of the Invention

The present invention relates to global regulators of bacterial pathogenic genes, and their use to confer disease resistance.

## 2. Description of the Related Art

A bibliography follows at the end of the Detailed Description of the Invention. The listed references are all incorporated herein by reference.

Cell-to-cell communication via small signal molecules is not only of vital importance to multi-celled living organisms such as animals and plants, it also plays important roles in the functional co-ordination among family members of single-celled organisms like bacteria. Rapid progress over the last few years has clearly established that N-acetyl-homoserine lactones, known as autoinducers

(AIs), are widely conserved signal molecules in Gram-negative bacteria. AIs were first found in marine bacteria *Vibrio* species in regulation of bioluminescence (Eberhard, et al., 1981; Cao and Meighen, 1989). In recent years, AIs have been identified in a wide range of Gram-negative bacteria. It has been found that AIs are involved in the regulation of a range of biological functions, including Ti plasmid conjugal transfer in *Agrobacterium tumefaciens* (Zhang, et al., 1993), induction of virulence genes in *Erwinia carotovora*, *Pseudomonas aeruginosa*, *Erwinia stewartii*, *Xenorhabdus nematophilus*, *Erwinia chrysanthemi*, *Pseudomonas solanaceum*, and *Xanthomonas campestris* (Jones, et al., 1993; Passador, et al., 1993; Pirhonen, et al., 1993; Pearson, et al., 1994; Beck von Bodman and Farrand, 1995; Barber, et al., 1997; Clough, et al., 1997; Costa and Loper, 1997; Dunphy, et al., 1997; Nasser, et al., 1998), regulation of antibiotics production in *Pseudomonas aureofaciens* and *Erwinia carotovora* (Pierson, et al., 1994; Costa and Loper, 1997), regulation of swarming motility in *Serratia liquifaciens* (Eberl, et al., 1996), and biofilm formation in *Pseudomonas fluorescens* and *P. aeruginosa* (Allison, et al., 1998; Davies, et al., 1998). Many more bacterial species are known to produce AIs but the biological functions related have not been established yet (Bassler, et al., 1997; Dumenyo, et al., 1998; Cha, et al., 1998).

Different bacterial species could produce different AIs. All AI derivatives share identical homoserine lactone moieties but can differ in the length and the structure of their acyl groups. The key

components in AI-mediated gene regulation systems are LuxI and LuxR type proteins. It has been established now that LuxI-type protein serves as an autoinducer synthase that utilizes acyl-ACPs and AdoMet (S-adenosylmethionine) as substrates (More, *et al.*, 1996; Schaefer, *et al.*, 1996). LuxR-type protein is proposed to be both a receptor for AIs and a AI-dependent transcriptional regulator that binds DNA immediately upstream of the *lux* promoter (Meighen, 1994; Sitnikov, *et al.*, 1995). A 20-nucleotide inverted repeat has been identified which is centered 44 nucleotides upstream of the transcription start site of the luminescence operon. This sequence called *lux* box is required for transcriptional activation by LuxR and is probably the LuxR binding site (Fuqua, *et al.*, 1994). Similar 18-bp *tra* boxes are found upstream of at least three *TraR*-regulated promoters, and disruption of these elements abolishes transcriptional activation by *TraR* (Fuqua and Winans, 1996a).

LuxR-type proteins appear to be composed of two modules (Choi and Greenberg, 1991; Hanzelka and Greenberg, 1995). Their carboxyl terminal regions contain a conserved short sequence of 19-amino acid, putative probe-type helix-turn-helix motif, predicted to be involved in binding to target promoters. A general mechanism of activation has been proposed by which the N-terminal domain of LuxR-type protein acts negatively to prevent an interaction between its C-terminal domain and the target DNA binding sites. This inhibition can be relieved by the action of an autoinducer ligand. A strong piece of evidence is that deletion of the N-terminal domain of LuxR results in constitutively active alleles of *luxR*, whereas larger

deletions that remove part of the predicted DNA binding domain abolish transcriptional activation (Choi and Greenberg, 1991). However, other members might use different mechanisms. Recent genetic studies indicate  
5 that EsaR and ExpR are likely to be repressors of their target genes rather than activators. Expression of the genes that are repressed by EsaR and ExpR is increased by autoinducers (Beckvon Bodman and Farrand 1995; Throup, et al. 1995). It appears that binding of these  
10 proteins to their target sites in promoter region causes repression, therefore autoinducer ligands may act to reduce binding affinity.

Evidence that the autoinducer binding site resides in the amino terminal domain of the LuxR protein has  
15 been presented (Hanzelka and Greenberg, 1995). LuxR alleles that have mutated amino terminal region require higher level of this signal that does the wild type, indicating this region required for ligand interaction (Slock, et al., 1990; Shadel, et al., 1990). This  
20 region (aa 79-127) and a region within the DNA-binding domain (aa 180-230) show a higher degree of conservation among LuxR and its homologs (ca 50% identity) than other parts of these polypeptides. However, the proposed protein-ligand interaction  
25 between LuxR and autoinducer has not been proved yet. Analysis of merodiploid *E. coli* strains containing wild-type and mutant LuxR alleles suggested that LuxR functions as a homomultimer and that a region required for multimerization resides within amino acid residues  
30 116 and 161 (Choi and Greenberg, 1992).

## BRIEF SUMMARY OF THE INVENTION

In one aspect, the present invention relates to an isolated nucleic acid molecule encoding a bacterial autoinducer inactivation protein.

5 In another aspect, the present invention relates to a expression vector which comprises a nucleic acid molecule encoding a bacterial autoinducer inactivation protein, wherein the expression vector propagates in a procaryotic or eucaryotic cell.

10 In yet another aspect, the present invention relates to a cell of a procaryote or eucaryote transformed or transfected with the expression vector of the present invention.

15 In yet another aspect, the present invention relates to an isolated protein which has bacterial autoinduction inactivation activity, where the protein comprises the amino acid sequence of SEQ ID NO: 2.

20 In yet another aspect, the present invention relates to a method for increasing disease resistance in a plant or animal, which method comprises introducing into a cell of such plant or animal a nucleic acid sequence which encodes a bacterial autoinducer inactivation protein in a manner which allows said cell to express said nucleic acid sequence.

25 In yet another aspect, the present invention relates to a method of preventing or reducing bacterial damage to a plant or animal, which method comprises administering to a plant or animal in need of such prevention or reduction an effective amount of a bacterial autoinducer inactivation protein.

30 In yet another aspect, the present invention relates to a composition for reducing bacterial damage to a plant or animal, which comprises:

- a) an effective amount of a bacterial autoinducer inactivation protein; and
- b) a suitable carrier.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the time course of AIs inactivation by cell extracts from *Bacillus* sp. strain 240BI. Cell extracts in 0.2 M phosphate buffer (pH 7.0) containing 100 ug total protein were added to the same buffer containing OHHL in a final concentration of 20 uM. The reaction was conducted in a 1.5 ml Eppendorf centrifuge tube in a final volume of 200 microliters and incubated at 28°C. Same concentration of OHHL in the phosphate buffer was used as control. Samples were taken at 10-min interval till 60 min and the reaction was stopped by boiling for 3 min. The samples were centrifuged for 5 min in a bench top centrifuge at the top speed and then assayed for AIs activity as described (Zhang, 1993). Blue colony indicates the presence of AI that activates the lacZ reporter gene, and white colony indicates absence of AI. Rows from left to right: 1, OHHL control without protein extract; 2 - 7, samples after 10, 20, 30, 40, 50, 60 min enzyme reaction.

Figure 2 shows the estimation of molecular mass of AIs inactivation enzyme. A 600 µl aliquot of cell extracts was added to the Centricon 30 (Amicon) and was centrifuged at a speed of 5000 x g for 30 min at 4°C. Passing fraction (550 microliters) and un-passing fraction (50 microliters) were topped up separately to a final volume of 600 microliters by adding 0.2 M phosphate buffer (pH 7.0). For bioassay, different



amounts of protein samples were added to the tubes containing OHHL in a final concentration of 20  $\mu$ M. From row 1 to 6, protein samples added were 2, 4, 6, 8, 10 and 0  $\mu$ l and the final reaction volume was 20 microliters for each reaction. Plate A: Passing fraction, Plate B: un-passing fraction.

Figure 3 shows the cloning and deletion analysis of *Bacillus* SP. strain 240B1 AI inactivation region. Cosmid clone E7-R3 contains the 4.3-kb *EcoRI* fragment identified by restriction analysis of overlapping cosmid clones. For deletion analysis, the same fragment was cloned into cloning vector pGEM-7Zf(+) for generation of clone E7-7. The deletion subclones were produced by restriction enzyme digestion and Dnase I treatment from the clone E7-7. The location and direction of *Ptac* promoters in the cosmid and in the pGEM-7Zf(+) clone are indicated by arrows. AI inactivation activity of the clones is shown in the second column: +, with AI inactivation activity; -, without AI inactivation activity. Restriction enzymes: E, *EcoRI*; H, *HindIII*; Ev, *EcoRV*; St, *StyI*. The location and direction of transcription of the *aiiA* ORF is indicated by an open arrow.

Figure 4A shows the nucleotide sequence of the *aiiA* gene [SEQ ID NO:1]. The potential ribosome binding sequence and -10 promoter element are underlined and double underlined respectively. The coding portion starts at base 1. The putative factor-independent termination site is labeled by a thick underline. Figure 4B shows the predicted amino acid sequence of the *aiiA* gene product [SEQ ID NO:2].

A short peptide sequence similar to the aspartyl protease active site consensus motif is underlined.

Figure 5 shows the best match of amino acids sequence of *aiiA* gene product (*AiiA*) to the consensus aspartyl proteases active site motif (Asp). Symbol: X, any amino acid. A vertical line indicates perfect match.

Figure 6 shows the bioassay for Als inactivation activities in *Bacillus* sp. strain 240B1, *E. coli* clones and AIs production activity in *Erwinia carotovora* strains. Row 1, OHHL control; row 2, *Bacillus* sp. strain 240B1; row 3, *E. coli* DH5 $\alpha$ ; row 4, *E. coli* DH5 $\alpha$  (pE7-R3); row 5, *E. coli* DH5 $\alpha$  (pF41); row 6, *Erw. carotovora* SCG1 (pE7R3); row 7, *Erw. carotovora* SCG1 (pLAFR3); row 8, *Erw. carotovora* SCG1. In the bioassay, OHHL was added to a final concentration of 20  $\mu$ M to the samples from lines 1 to 5. No exogenous AIs were added to the samples from rows 6 to 8.

Figure 7 shows the effect of *aiiA* gene expression in *Erw. carotovora* on pathogenicity in (A), potato; (B), eggplant; (C), Chinese cabbage; (D), carrot; and (E), celery. Top: plant tissues were inoculated with *Erw. carotovora* SCG1. Bottom: plant tissues were inoculated with *Erw. carotovora* SCG1 (pE7-R3). The actively growing bacteria were centrifuged for 1 min at 3000 x g, resuspended with YEB liquid medium to OD600 = 1.3 ( $2 \times 10^9$  cfu/ml) which was designed as  $10^0$  inoculum. The  $10^0$  inoculum was diluted 5 and 10 times respectively to prepare  $10^{-1/2}$  and  $10^{-1}$  dilutions. The Plant tissues were inoculated by adding a 4- $\mu$ l volume of bacteria

inoculum to the freshly cut surface or a wounding site punched by a pipette tip. The inoculum concentration from the left to the right plate:  $10^0$ ;  $10^{-1/2}$ ; and  $10^{-1}$ . The inoculated plant tissues were placed in plastic plates and incubated at  $28^\circ\text{C}$ . The photograph was taken 48 h after inoculation.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that the SEQ ID NO:2 protein has the effect of reducing or eliminating the activity of bacterial autoinducers (AIs). Consequently, the protein, and any nucleic acid that encodes the protein, may be used in a variety of situations where it is desired to reduce or eliminate the effect of such bacteria.

In one preferred aspect, the present invention provides a nucleic acid molecule which is selected from the group consisting of:

a) a nucleic acid having the sequence of SEQ ID NO:1;

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:2; and

c) a nucleic acid that hybridizes to a) or b) above, wherein a positive hybridization signal is observed after washing with 1 X SSC and 0.1% SDS at  $55^\circ\text{C}$  for one hour. The nucleic acid optionally further comprises a signal peptide coding region of any sequence.

The nucleic acid sequence may be used to confer bacterial resistance in plants or animals. A nucleic acid that encodes a bacterial autoinducer inactivation protein can be introduced into a cell such that the

inactivation protein is expressed by the plant or animal.

The nucleic acid sequence may be used to confer resistance to diseases where the expression of pathogenic genes are regulated by autoinducers, such as the diseases caused by *Pseudomonas aeruginosa*, *Erwinia stewartii*, *Xenorhabdus nematophilus*, *Erwinia chrysanthemi*, *Pseudomonas solanaceum*, and *Xanthomonas campestris* (Passador, et al., 1993; Pirhonen, et al., 1993; Pearson, et al., 1994; Beck von Bodman and Farrand, 1995; Barber, et al., 1997; Clough, et al., 1997; Costa and Loper, 1997; Dunphy, et al., 1997; Nasser, et al., 1998). Preferably, in the agricultural setting, the sequence may be used to confer soft rot disease resistance in susceptible plants, such as potato, eggplant, Chinese cabbage, carrot and celery.

The sequence may be introduced into plant or animal cells by well-known methods. Methods for the transformation or transfection of eukaryotic cells with exogenous nucleic acid sequences include transfection, projectile bombardment, electroporation or infection by *Agrobacterium tumefaciens*. These methods are likewise familiar to the person skilled in the area of molecular biology and biotechnology and need not be explained here in detail. As pathogenic bacteria cells are confined to the intercellular area of plant tissues, it is desirable to target the AiiA protein into the intercellular spaces. Such may be accomplished by fusing a secretion signal peptide to the AiiA protein (Sato, et al., 1995; Firek, et al., 1993; Conrad and Fiedler, 1998; Borisjuk, et al., 1999). Alternatively, a plant membrane attachment motif can be incorporated into the peptide sequence of AiiA for anchoring the

AiiA enzyme in the outer surface of plant cell membrane.

The present invention provides a new strategy for engineering resistance to diseases. In particular, this strategy targets N-acyl homoserine lactone autoinducers that induce expression of pathogenic genes of many bacterial pathogens at a threshold concentration. This strategy is applicable to all plant, animal or mammal diseases where the expression of pathogenic genes of the bacterial pathogens is inducible by N-acyl homoserine lactone autoinducers.

The present invention also contemplates usage of a bacterial autoinducer inactivation protein directly to treat or prevent bacterial damage. For example, the protein may be applied directly to plants in need of such treatment or prevention. In a preferred embodiment, the protein is applied in the form of a composition which comprises an effective amount of the protein and a suitable carrier. The composition may have a wide variety of forms, including solutions, powders, emulsions, dispersions, pastes, aerosols, etc.

The bacterial autoinducer inactivation protein may also be used to treat bacterial infections in animals, including humans. In that application, an effective amount of the active ingredient is administered to an animal in need of such treatment.

For therapeutic treatment, the active ingredient may be formulated into a pharmaceutical composition, which may include, in addition to an effective amount of the active ingredient, pharmaceutically acceptable carriers, diluents, buffers, preservatives, surface active agents, and the like. Compositions may also

include one or more other active ingredients if necessary or desirable.

The pharmaceutical compositions of the present invention may be administered in a number of ways as will be apparent to one of ordinary skill in the art. Administration may be done topically, orally, by inhalation, or parenterally, for example.

Topical formulations may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Oral formulations include powders, granules, suspensions or solution in water or non-aqueous media, capsules or tablets, for example. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be used as needed.

Parenteral formulations may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

The dose regimen will depend on a number of factors which may readily be determined, such as severity and responsiveness of the condition to be treated.

Aspects of the invention will now be illustrated with reference to the following non-limiting examples.

#### EXAMPLE 1

Bacterial isolate 240B1 was isolated from soil suspension based on its function for inactivation of N- $\beta$ -oxo-hexanoyl-L-homoserine lactone (OHHL) and N- $\beta$ -oxooctanoyl-L-homoserine lactone (OOHL) and N- $\beta$ -oxodecanoyl-L-homoserine lactone (ODHL) (Zhang, et al., 1993). Unless otherwise stated, OHHL was used for routine bioassay. *Erwinia carotovora* strain SCG1 was isolated from Chinese cabbage leaf showing soft rot

symptoms. It has been confirmed that strain SCG1 produces AIs and elicits soft rot disease in potato and Chinese cabbage. *Escherichia coli* strain DH5 $\alpha$  was used as a host for DNA cloning and subcloning.

- 5 *Agrobacterium tumefaciens* strain NT1 (*traR*; *tra::lacZ749*) was used as an indicator in bioassay for AI activity (Piper, et al., 1993). *E. coli* strain was cultured in Luria-Bertani (LB) medium at 37°C and other strains were cultured in LB (Miller, 1972) or YEB
- 10 medium (per liter contains: casein hydrolysate 10 g, yeast extract 5 g, NaCl 10 g, sucrose 5 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g, agar 15 g, pH 7.2) at 28 °C. The minimal salts medium with mannitol and (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as carbon and nitrogen sources was used for bioassay of OHHL (Petit
- 15 and Tempe, 1978). Appropriate antibiotics were added as indicated at the following concentrations: ampicillin, 100 µg/ml; tetracycline, 20 µg/ml and kanamycin, 50 µg/ml.

#### **Bioassay of AIs activity**

- 20 The qualitative and quantitative bioassay methods for determination of AIs activity has been described previously (Zhang, 1993). For determination of the AIs production ability of wild-type and genetically modified *Erwinia* strains, the same bioassay procedure
25. was used except that no OHHL was added into the bacterial culture.

#### **Cloning and sequencing of the AiiA gene**

- Genomic DNA from 240B1 was digested partially with *EcoRI*. DNA fragments were ligated to the
- 30 dephosphorylated *EcoRI* site of cosmid vector pLAFR3 (Staskawicz, et al., 1987). Ligated DNA was packaged

with Gigapack III XL Packaging Extract (Stratagene) and transfected into *E. coli* DH5 $\alpha$ . Cosmid clones with OHHL inactivation activity were identified by using the bioassay method described above. Subcloning into sequencing vector pGEM-7Zf(+) was carried out by routine techniques (Sambrook, et al., 1989). Deletion analysis was carried out by using DnaseI method as described by Lin, et al. (1985). The sequencing was performed on both strands using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Nucleic acid sequence data and deduced amino acid sequences were analyzed with a DNASTAR™ sequence analysis software package (DNASTAR Inc.) and database searches were performed using the BLASTA search algorithm (Altschul, et al., 1990).

#### ***Genetic modification of Erwinia strain SCG1***

The E7-R3 plasmid, carrying the *aiiA* gene in the cosmid vector pLAFR3, was transferred into *Erwinia* stain SCG1 by triparental mating with the helper strain RK2013 (Ditta, et al., 1980). Transconjugants were selected on the plates containing minimal medium with tetracycline and confirmed by PCR with primers specific to the *aiiA* gene.

#### ***Virulence tests***

The virulence of wild-type *Erw. carotovora* strain SCG 1 and the *aiiA* gene transformant SCG1(E7-R3) was evaluated by inoculation. Four  $\mu$ l of early stationary phase bacterial suspension (containing  $\sim 2 \times 10^9$  cell/ml) or diluted bacteria was added to the cut surfaces or wounding sites of plant tissues. The inoculated plant



tissues were incubated in a Petri dish at 28°C overnight. The severity of soft rot was examined 48 hours after incubation.

## Results

### 5 *Screening of bacteria that inactivate AIs*

Bacterial isolates from plant and soil samples were screened for enzymatic inactivation of AIs. A bacterial isolate 240B1, which showed a strong ability to eliminate AIs activity, was selected for further study. The total protein extracts from isolate 240B1 eliminated AIs activity completely during one-hour incubation (Fig. 1), and the capacity of the protein extract to inactivate AIs was abolished by treatment with proteinase K for 1 hour or boiling for 5 min. These observations indicate enzymatic inactivation of AIs by bacterial isolate 240B1. The isolate was taxonomically characterized as *Bacillus* sp., because of the following characteristics: Gram-positive, rod-shaped, catalase positive, facultatively anaerobic, and 16 rRNA sequence homology to that of other *Bacillus* bacteria (data not shown).

The molecular mass of the enzyme for AIs inactivation appears to be larger than 30 kDa. Its activity was lost after passing the protein extract through Centricon 30 (Amicon) but the activity was recovered in the re-suspended fraction that failed to pass the Centricon 30 (Fig. 2).

### *Cloning and localization of AIs inactivation region*

To identify the gene encoding AIs inactivation, a cosmid library was constructed with the genomic DNA of *Listera* sp. strain 240B1. Twelve hundred clones were

screened for AIs inactivation activity. Three clones showing AIs inactivating function were identified. Restriction analysis showed that the 3 clones shared one common band of 4.3-kb generated by *EcoRI* digestion.

5 The bioassay with the subclone E7-7 containing this 4.3-kb *EcoRI* fragment confirmed that this fragment encodes AIs inactivation function (Fig. 3). To identify the minimum size and the location of the AIs inactivation gene (*aiiA*), a serial of deletion clones  
10 was generated by deletion from both ends of this 4.3-kb fragment with the DNaseI method (Lin, et al., 1985). The results indicated that the *aiiA* gene is contained in a 1.2 Kb fragment in clone F41 (Fig.3).

#### ***AiiA gene encodes a novel protein***

15 The 1.2-kb DNA insert in clone F41 was completely sequenced from both strands. The nucleotide sequence of *aiiA* and the predicted amino acid sequence are shown in Fig. 4. The complete sequence of the DNA insert contains 1,222 base pairs and there are 4 potential  
20 in-frame open reading frames (ORF) starting from nucleotide position of 1, 42, 156 and 228 respectively (Fig. 4). Deletion analysis indicated that only the longest ORF encodes AIs inactivation function, because the clone R34, in which the 48 bp promoter region and  
25 nucleotides from 1 to 13 in the longest ORF were deleted, lost AI inactivation function completely, although the remaining DNA insert was placed under the control of a functional *Ptac* promoter (Fig. 3). This is confirmed by fusing the longest ORF to the  
30 glutathione S-transferase gene in the same ORF and testing for AI inactivation activity of the purified fusion protein (data not shown). This ORF contains 750

bp nucleotide and encodes a protein of 250 amino acids, with a predicted molecular mass of 28,036 daltons and an isoelectric point at 4.7, because of 19 strongly basic and 39 strongly acidic amino acids residues. The putative initiation codon is preceded at a spacing of 7 bp by a potential ribosome-binding sequence (AAGGTGG) which is complementary to the 3' end of the *E. coli* 16S rRNA. The best sequence match (TATTGT) to the consensus -10 promoter element (TATAAT) occurs 35 bp upstream of the initiation codon. A TCTT box following a T-rich region resembling the potential factor-independent termination site is found downstream of the termination codon (Brendel, 1986). The total GC content of the *aiiA* gene is 37% and GC content in the third position of the codon is 27.2%.

Database searches showed that the *aiiA* gene has no significant similarity to known sequences in the major databases (GenBank, European Molecular Biology Laboratory, Protein Information Resource, and Swiss-Prot) by FASTA and BLAST analysis at either nucleotide or peptide sequence level, suggesting that *AiiA* is a novel protein. Consensus protein motif search using the Genetics Computer Group (Madison, WI) MOTIF program showed that a short peptide sequence, "ILVDTGMPESAV" from position 47 to 58 in *AiiA*, is similar but not identical to the aspartyl protease active site signature pattern (Rawlings and Barrett, 1995) (Fig. 5).

#### ***Expression of aiiA gene in Erwinia carotovora decreases AIs releasing and attenuates virulence***

The cosmid clone E7-R3 was transferred into *Erwinia carotovora* strain SCG1 by triparental mating. The pLAFR3 vector has been safely maintained in *Erwinia*

carotovora without selection pressure. The bioassay showed that the AIs released by *Erwinia carotovora* (E7-R3) was significantly reduced (Fig. 6, lane 6), while the presence of the cosmid vector pLAFR3 alone in *Erwinia carotovora* did not affect AIs production (Fig. 6, lanes 7). Data suggest that the most of AIs produced by *Erwinia carotovora* strain SCG1 was inactivated by *aiiA* gene product.

The *Erwinia carotovora* SCG1(E7-R3) that expresses *AiiA* protein failed to or caused only minor soft rot disease symptom in potato, eggplant, Chinese cabbage, carrot and celery, while its parental strain caused severe symptoms (Fig. 7A, B, C, D, E). To prevent experimental errors due to genetic variations, four colonies from *Erwinia carotovora* strain SCG1 and its *aiiA* gene transformants respectively, were randomly selected for testing AIs production and virulence on potato. Similar results were obtained in both experiments. The *Erwinia carotovora* strain SCG1 (pLAFR3) that contains the cosmid vector only caused the same level of disease severity as its parental strain *Erwinia carotovora* strain SCG1 (Fig. 7F).

## Discussion

Bacterial isolate 240B I, which was identified as *Bacillus* sp., produces an enzyme that can effectively inactivate the three AIs tested, i.e., N- $\beta$ -oxo-hexanoyl-L-homoserine lactone, N- $\beta$ -oxo-octanoyl-L-homoserine lactone and N- $\beta$ -oxo-decanoyl-L-homoserine lactone. The gene (*aiiA*) encoding the AI inactivation enzyme has been cloned and fully sequenced. Expression of the *aiiA* gene in transformed *E. coli* and pathogenic bacteria *Erwinia carotovora* confers ability for AI

inactivation and significantly reduces the AIs release from *Erwinia carotovora*. To our knowledge, it is the first protein identified capable of enzymatic inactivation of N-acyl-homoserine lactones, the autoinducers for global gene regulation in a diverse of bacteria species.

The AiiA is a novel protein. There is no significant homology to known proteins in major databases. It shares similarities to the consensus pattern of the aspartyl proteases active site (Rawlings and Barret, 1995). Aspartyl proteases, also known as acid proteases, are widely distributed in vertebrates, fungi, plants, retroviruses and some plant viruses. The aspartyl proteases from most retroviruses and some plant viruses are homodimers. The molecular mass of AiiA protein is about 28 kDa but it failed to pass a molecular sieve with a cut off size of 30 kDa, indicating a possibility that AiiA protein exists as a homodimer or homomultimer under the natural conditions. However, there is also a possibility that AiiA monomer has an irregular three-dimensional structure, which hinders it passing through the molecular sieve. Aspartyl proteases are endopeptidases and hydrolyses amide linkages of proteins. Crystallographic study has shown that the enzyme of the aspartyl protease family are bilobed molecules with the active-site cleft located between the lobes, and each lobe contributing one of the pair of aspartic acid residues that is responsible for the catalytic activity (Sielecki et al., 1991).

*Erwinia carotovora* is a plant pathogen that produces and secretes exoenzymes that act as virulence determinants for soft rot diseases of various plants

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including potato, cabbages, tomato, chili, carrot, celery, onion, and lettuce (Kotoujansky, 1987).

Mutants that were defective in the producing N-3-(oxohexanoyl)-L-homoserine lactone were also

5 defective in synthesis of the pectinase, cellulase and protease exoenzymes. These mutants failed to induce soft rot disease in potato tubers (Jones, et al., 1993). It was found that the *expI* gene, which is homologous to *luxI* gene of *Vibrio fischeri*, encodes  
10 autoinducer production in *Erwinia carotovora*. The *expI* mutant was avirulent when it was inoculated to tobacco leaf but the virulence was restored by external autoinducer addition (Pirhonen, et al., 1993).

Obviously, autoinducers are a potential target for  
15 genetic engineering of plant soft rot disease resistance. As an interim test and a concept proving approach, the cosmid clone containing the *aiiA* gene was introduced to *Erwinia carotovora* strain SCG1.

Expression of the AiiA enzyme in *Erwinia carotovora*  
20 significantly reduced the release of autoinducers, and the genetically modified *Erwinia carotovora* that expressed AiiA failed to induce any or induce only minor soft rot disease symptom on all plants tested, including potato, eggplant, Chinese cabbage, carrot and  
25 celery. Our results further support the important role of autoinducers in the regulation of expression of virulence genes in *Erwinia carotovora*, and the potential of the *aiiA* gene to confer resistance to soft rot disease and other diseases in which the  
30 autoinducers are involved in regulation of pathogenic gene expression.

The present invention provides a new strategy for engineering resistance to diseases. In particular,

this strategy targets N-acyl homoserine lactone autoinducers that induce expression of pathogenic genes of many bacterial pathogens at a threshold concentration. By using the above-mentioned conception-proving approach, the present invention demonstrates that reduction or elimination of autoinducers produced by pathogenic bacteria by an autoinducer inactivation enzyme significantly attenuates pathogenicity of otherwise virulent bacterial pathogen. Because the expression of pathogenic genes in pathogenic bacteria requires a threshold concentration, this AI-inactivation strategy is applicable to all plant, animal or mammal diseases where the expression of pathogenic genes of the bacterial pathogens is inducible by N-acyl homoserine lactone autoinducers.

The *aiiA* gene could also be a useful tool for investigation of the role of AIs in those bacteria where the biological functions regulated by AIs has not been established. In recent years, many more bacteria species have been shown to produce AIs (Bassler, et al., 1997; Dumenyo, et al., 1998; Cha, et al., 1998; Surette, et al., 1999). Some of them are important plant pathogens such as *Pseudomonas* and *Xanthomonas* species. The gene knock out approach based on sequence homology could be difficult. The overall levels of sequence similarity of AIs synthase and the related regulatory protein from different genera are rather low, often no higher than 28-35% identity between LuxI-type proteins and 18-25% identity for LuxR-type proteins (Fuqua et al., 1996). However, it is feasible and simple to introduce the *aiiA* gene into these

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bacteria to probe the biological functions regulated by AIs.



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## CLAIMS

1. An isolated nucleic acid molecule encoding a bacterial autoinducer inactivation protein.

2. The molecule of claim 1, wherein the nucleic acid molecule is selected from the group consisting of:

a) a nucleic acid having the sequence of the coding portion of SEQ ID NO:1;

b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:2; and

c) a nucleic acid that hybridizes to a) or b) above, wherein a positive hybridization signal is observed after washing with 1 X SSC and 0.1% SDS at 55°C for one hour.

3. The molecule of claim 1, which further comprises a signal peptide coding region of any sequence.

4. An expression vector which comprises the nucleic acid molecule of claim 1, wherein the expression vector propagates in a procaryotic or eucaryotic cell.

5. A cell of a procaryote or eucaryote transformed or transfected with the expression vector of claim 4.

6. An isolated protein which has bacterial autoinduction inactivation activity, where the protein comprises the amino acid sequence of SEQ ID NO: 2.

7. A method for increasing disease resistance in a plant or animal, which method comprises introducing

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into a cell of such plant or animal a nucleic acid sequence which encodes a bacterial autoinducer inactivation protein in a manner which allows said cell to express said nucleic acid sequence.

5           8. The method of claim 7, wherein the nucleic acid sequence is selected from the group consisting of:

          a) a nucleic acid having the sequence of the coding portion of SEQ ID NO:1;

10           b) a nucleic acid encoding the amino acid sequence of SEQ ID NO:2.

          9. The method of claim 7 or 8, wherein the nucleic acid sequence further comprises a signal peptide coding region of any sequence.

15           10. The method of claim 7 or 8, wherein the nucleic acid sequence further comprises a membrane attachment domain-coding region of any source.

          11. The method of claim 7, wherein the plant is susceptible to bacterial soft rot disease.

20           12. The method of claim 11, wherein the plant is selected from the group consisting of potato, eggplant, Chinese cabbage, carrot and celery.

          13. The method of claim 7, wherein the plant is susceptible to a bacterial disease in which the expression of a virulence gene is regulated by an N-acyl homoserine lactone autoinducer.

25

          14. A method of preventing or reducing bacterial damage to a plant or animal, which method comprises

administering to a plant or animal in need of such prevention or reduction an effective amount of a bacterial autoinducer inactivation protein.

5 15. The method of claim 14, wherein the protein comprises SEQ ID NO: 2.

16. A composition for preventing or reducing bacterial damage to a plant or animal, which comprises:

- 10 a) an effective amount of a bacterial autoinducer inactivation protein; and  
b) a suitable carrier.

17. The composition of claim 16, wherein the protein comprises SEQ ID NO: 2.

18. A method for screening of bacterial isolates for autoinducer inactivation activity, which comprises:

- 15 a) isolating a single colony bacterial culture from soil or plant samples;  
b) screening the culture for autoinducer inactivation activity;  
c) preparing a crude protein extract from the  
20 culture; and  
d) confirming enzymatic inactivation of autoinducer activity by the crude protein extract.

19. A method of isolating the nucleic acid of claim 1 or claim 2, which comprises the steps of:

- 25 a) preparing a gene bank from a donor organism that contains a nucleic acid sequence coding for a protein with an autoinducer inactivation activity in a suitable host organism;  
b) screening the clones of the gene bank; and

c) isolating the clones which contain a nucleic acid coding for a protein with autoinducer inactivation activity.

20. A process as claimed in claim 19, wherein *E. coli* is used as host organism.

21. A process as claimed in claim 19, wherein the steps of preparing a gene bank, screening the clones, and isolating the clones are performed in an *E. coli* strain that does not inactivate the autoinducer.

22. A method which comprises:

- a) introducing the nucleic acid sequence of claim 1 or claim 2 into a bacterial cell; and
- b) screening the bacterial cell obtained from step a) for changed biological function.

23. The method of claim 22, wherein the changed biological function is a function which is lost as a result of step a).

24. The method of claim 22, wherein the changed biological function is a function which is suppressed as a result of step a).

25. The method of claim 22, wherein the changed biological function is a function which is enhanced as a result of step a).

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(57) Abstract: Disclosed are an isolated nucleic acid molecule encoding a bacterial autoinducer inactivation protein, the encoded protein, and anti-bacterial uses therefor.

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Figure 1

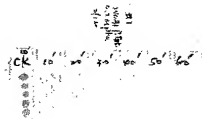


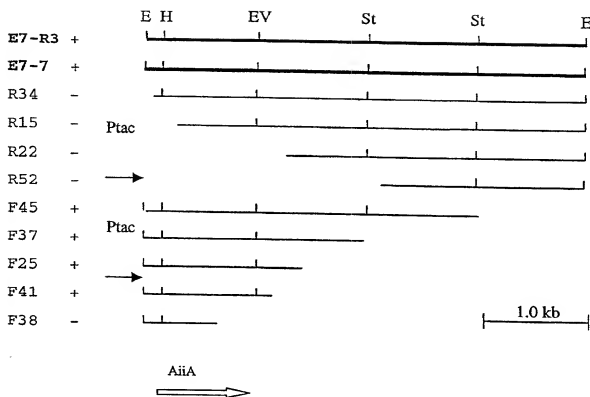
Figure 2





Figure 3

## Clone Activity



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Figure 4(A)

. .ctttactgtattgtttttattcaaaactaaatgtaaagggtggatacata -1

ATGACAGTAAAGAAGCTTTATTTCGTCCCAGCAGGTCGTTGTATGTTGGA 50

TCATTTCGTCTGTTAATAGTACATTAACACCAGGAGAATTATTAGACTTAC 100

CGGTTTGGTGTTATCTTTTGGAGACTGAAGAAGGACCTATTTTAGTAGAT 150

ACAGGTATGCCAGAAAGTGCAGTTAATAATGAAGGTCTTTTAAACGGTAC 200

ATTTGTGCGAAGGGCAGGTTTACCAGAAAATGACTGAAGAAGATAGAATCG 250

TGAATATTTTAAACGGGTTGGTTATGAGCCGGAAGACCTCTTTTATATT 300

ATTAGTTCTCACTTGCATTTTGATCATGCAGGAGGAAATGGCGCTTTTAT 350

AAATACACCAATCATTTGTACAGCGTGCTGAATATGAGGCGGCGCAGCATA 450

GCGAAGAAATATTTGAAAGAATGTATATTGCCGAATTTAAACTACAAAATC 500

ATTGAAGGTGATTATGAAGTCGTACCAGGAGTTCAATTATTGCATACACC 550

AGGCCATACTCCAGGGCATCAATCGCTATTAATTGAGACAGAAAAATCCG 600

GTCCTGTATTATTAAACGATTGATGCATCGTATACGAAAGAGAATTTTGAA 650

AATGAAGTGCCATTTGCGGGATTGATTCAGAATTAGCTTTATCTTCAAT 700

TAAACGTTTAAAGAAGTGGTGATGAAAGAGAAGCCGATTGTTTCTTTG 750

GACATGATATAGAGCAGGAAAGGGGATGTAAAGTGTTCCCTGAATATATA 800

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TAGtacaaaaagtcatgagccttattcgctcatgactttttcgtttaaatg 850  
 Atttttttaaaataagttataaaacttttttagaactatcttcatttaattg 900  
 Atagtacgtaagggtttacatcattaggagtatcttgttgagcaatcatca 950  
 Cttcgttactgtgatgggtcaactacccatatgaaatattttttataagtc 1000  
 Ccatcctcgaaagtaatccacatatcacagtctattaaatctgatccttc 1050  
 Ttcatcctaagttaattttccttttttggcgggtatccatactgttaatga 1100  
 Atgtttttaattcatctgtttttgtgagaaagatatccttttttgtttta 1150  
 Attgactcgacatgtatatcttttatttcttgttttcctaaaaagacagg 1200  
 gggctcatttgggtctcttttgagt 1222

Figure 4(B)

MTVKKLYFVPAGRCMLDHSSVNSTLTPGELLDLFPVWCYLLTEEGPILVD 50  
 TGMPESAVNNEGLFNQTFVEGQVLPKMTEDRIVNILKRVGYEPEDLLYI 100  
 ISSHLHFDHAGNGAFINTPIIVQRAEYEAQHSSEYLKECILPNLNYKI 150  
 IEGDYEVPVPGVQLLHTPGHTFGHQSLLIETEKSGPVLLTIDASYTKENFE 200  
 NEVPFAGFDSELALSSIKRLKEVVMKEKPIVFFGHDIQERGCKVFPEYI 250

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Figure 5

Asp: [LIVMFGAC] - [LIVMTADN] - [LIVFSA] - D - [ST] - G - [STAV] -  
 AiiA: I<sub>47</sub> L V D T G M

Asp: [STAPDENQ] - X - [LIVMFSTNC] - X - [LIVMFGTA]  
 AiiA: P E S A V<sub>58</sub>

Figure 6

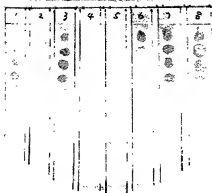
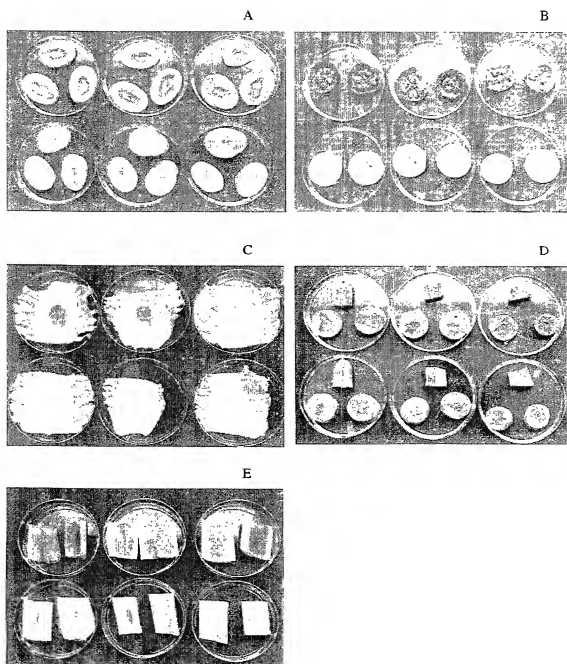


Figure 7



<p align="center"><b>DECLARATION AND POWER OF ATTORNEY FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)</b></p> <p> <input type="checkbox"/> Declaration Submitted with Initial Filing         <input checked="" type="checkbox"/> Declaration Submitted after Initial Filing       </p>		Attorney Docket No.	2577-127
		First Named Inventor	Lian-Hui ZHANG et al.
		COMPLETE IF KNOWN	
		Application Number	10/019,661
		Filing Date	January 2, 2002
		Group Art Unit	
		Examiner Name	

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **GLOBAL REGULATORS OF BACTERIAL PATHOGENIC GENES AS TARGETS FOR ENGINEERING DISEASE RESISTANCE** the specification of which was filed on January 2, 2002 (as PCT International Application Number PCT/SG9900128, filed November 17, 1999), now U.S. Application No. 10/019,661, and was amended on .

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

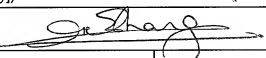

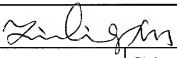
Prior Foreign Application Numbers	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
				YES	NO
SG 9903146-0	Singapore	07/02/1999	<input type="checkbox"/> <input type="checkbox"/>	<input type="checkbox"/> <input type="checkbox"/>	<input checked="" type="checkbox"/> <input type="checkbox"/>

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

I or we hereby appoint the registered practitioner(s) associated with Customer No. 6449 to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith. Direct all correspondence to Customer Number 6449.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

<b>NAME OF SOLE OR FIRST INVENTOR:</b>		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
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Inventor's Signature 		Date <u>17/04/2002</u>	
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City	State	Zip	Country Singapore
<b>NAME OF SECOND INVENTOR:</b>		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name <u>Yihu</u> (first and middle [if any])		Family Name <u>DONG</u> or Surname	
Inventor's Signature 		Date <u>17/04/2002</u>	
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City	State	Zip	Country Singapore
<b>NAME OF THIRD INVENTOR:</b>		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
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Mailing Address <u>SGX</u>			
City	State	Zip	Country Singapore
<b>NAME OF FOURTH INVENTOR:</b>		<input type="checkbox"/> A petition has been filed for this unsigned inventor	
Given Name (first and middle [if any])		Family Name or Surname	
Inventor's Signature		Date	
Residence: City	State	Country	Citizenship
Mailing Address			
Mailing Address			
City	State	Zip	Country